1.0 Draft ICCVAM Recommendations: The BG1Luc ER TA Test Method

Background and Introduction

ICCVAM is currently evaluating the validation status of the LUMI-CELL® BG1Luc4E2 ER TA test method (hereafter referred to as the BG1Luc ER TA test method), an *in vitro* method proposed to identify potential agonist or antagonist substances that might interfere with normal estrogen activity. Although the primary objective of this test method is to provide a qualitative assessment of *in vitro* estrogenic activity (i.e., positive or negative), quantitative analysis is also performed to provide additional information on the estrogenic activity of test substances. The BG1Luc ER TA uses BG-1 cells, a human ovarian carcinoma cell line that was stably transfected with an estrogen-responsive luciferase reporter gene to measure whether and to what extent a substance induces or inhibits TA activity via ER mediated pathways.

NICEATM led and coordinated an international validation study with its counterparts in Japan (JaCVAM) and Europe (ECVAM) to assess the accuracy and reliability of the BG1Luc ER TA test method for the qualitative detection of substances with *in vitro* ER agonist or antagonist activity. Based on the results of this study, ICCVAM developed draft test method recommendations on the usefulness and limitations of the BG1Luc ER TA for identifying potential estrogen agonists or antagonists. ICCVAM also developed draft recommendations for standardized test method protocols, future studies and performance standards.

The results of the validation study, along with additional information and discussion of the evaluation of this test method are provided in a draft ICCVAM background review document (BRD). An international independent scientific peer review panel (Panel) will convene in public forum March 29-30, 2011, to review the draft BRD for completeness and determine the extent to which it supports the draft ICCVAM test method recommendations for the BG1Luc ER TA test method.

1.1 Draft ICCVAM Recommendations: Test Method Uses and Limitations

1.1.1 Evaluation as a Screening Test to Identify Substances with Estrogen Agonist Activity

ICCVAM proposes that the BG1Luc ER TA test method can be used as a screening test to identify substances with *in vitro* estrogen agonist activity. This use is based on an evaluation of available validation study data and corresponding accuracy and reliability. ICCVAM concludes that the accuracy of this assay is at least equivalent to the current ER TA included in regulatory testing guidance.

The supporting accuracy analysis using 35 ICCVAM reference substances that produced a definitive result in agonist testing indicated a concordance of 97% (34/35), sensitivity of 96% (27/28), specificity of 100% (7/7), false positive rate of 0% (0/7), and false negative rate of 4% (1/28) when compared with existing reference data from other *in vitro* ER TA assays. Only L-thyroxine was false negative in the BG1Luc ER TA test method when compared to the ICCVAM reference classification. This reference substance is classified as Positive (2/3) based on two reports of positive agonist activity and one report of no agonist activity. The two positive results were in GH3 cells (rat pituitary adenoma) and HeLa cells (human cervical carcinoma), where as MCF-7 cells (human breast adenocarcinoma) showed no estrogenic response when exposed to L-thyroxine. These reports indicate a possible tissue-specific response to this chemical, which may explain the lack of ER agonist activity observed in this experiment with BG-1 cells (human ovarian carcinoma).

Although the classifications for some of the 12 substances that were tested for agonist activity at least three times at each laboratory (test substances differed among the laboratories), there was 100% agreement *within* each laboratory for each of the three repeat tests. When comparing results *across* laboratories for these 12 substances, all three laboratories agreed on 67% (8/12) of the substances. An additional 36 substances tested once in each laboratory for agonist activity produced a definitive result in at least two laboratories. There was 100% agreement among the laboratories for 83% (30/36) of these substances.

The US EPA OPPTS 890.1300 / CERI STTA method for assessing ER-alpha agonist activity of test substances is currently the only ER TA test method accepted by regulatory agencies. Because the BG1Luc ER TA test method is another STTA that could be considered for regulatory use, a comparison of test method accuracy between these two test methods was conducted based on a list of ICCVAM-recommended agonist reference substances for which definitive classifications have been produced in both methods (**Section 5.4**). These results show identical levels of accuracy when both methods tested the same agonist reference chemicals: concordance 95% (24/25), sensitivity 95% (21/22), and specificity 100% (4/4). Overall, these data indicate that the BG1Luc ER TA is equivalent to the US EPA OPPTS 890.1300 method for assessing ER-alpha agonist activity.

Based on these results, the BG1Luc ER TA agonist test method can be applied to a wide range of substances, provided they can be dissolved in DMSO, do not react with DMSO or the cell culture medium, and are not toxic for the cells. Although this method may be applicable to mixtures, none were evaluated in this validation study. Volatile substances may yield acceptable results if CO_2 permeable plastic film is used to seal the test plates, but no volatile substances were evaluated in this validation study. Although relatively few are known, substances with endogenous luminescence or

that naturally inhibit luciferase activity cannot be used in this, or any other, luciferase-based test method. The demonstrated performance of the BG1Luc ER TA agonist test method suggests that data generated with this test method could be routinely considered for prioritization of substances for further testing.

1.1.2 Evaluation as a Screening Test to Identify Substances with Estrogen Antagonist Activity

Based on an evaluation of available data and corresponding performance (accuracy and reliability), ICCVAM recommends that the BG1Luc ER TA test method be used as a screening test to identify substances with estrogen antagonist activity. The accuracy analysis conducted with 25 reference substances that produced a definitive result in antagonist testing indicated an overall accuracy of 100% (25/25), sensitivity of 100% (3/3), specificity of 100% (22/22), false positive rate of 0% (0/22), and false negative rate of 0% (0/3).

Although the classifications for some of the 12 substances that were tested for antagonist activity at least three times at each laboratory (test substances differed among the laboratories), there was 100% agreement *within* each laboratory for each of the three repeat tests. When comparing results *across* laboratories for these 12 substances, there was 100% agreement among the three laboratories for all 12 substances. An additional 41 substances tested once in each laboratory for antagonist activity produced a definitive result in at least two laboratories. There was 100% agreement among the laboratories for 93% (38/41) of these substances.

Based on these results, the limitations of the BG1Luc ER TA antagonist test method appear to be the same as those identified for the agonist test method described above. Although the validation database is somewhat limited in number (n=25), the demonstrated performance of the BG1Luc ER TA antagonist test method suggests that data generated with this test method could be routinely considered for prioritization of substances for further testing. This is further supported by the fact that so few ER antagonists have been definitively identified, and all three tested in the BG1Luc ER TA antagonist test method were correctly identified.

1.2 Draft ICCVAM Recommendations: Test Method Protocol for the BG1Luc ER TA Test Method

For use of the BG1Luc ER TA test method as a screening test to identify substances with estrogen agonist or antagonist activity, ICCVAM recommends using the ICCVAM BG1Luc ER TA agonist and antagonist test method protocols that are appended to this report. In addition, all future studies intended to further characterize the usefulness and limitations of the BG1Luc ER TA agonist and antagonist test methods should be conducted using these recommended protocols.

1.3 Draft ICCVAM Recommendations: Future Studies for the BG1Luc ER TA Test Method

ICCVAM promotes the scientific validation and regulatory acceptance of new methods that refine, reduce, or replace animal use where scientifically feasible. The rat uterine cytosol ER binding assay, currently listed as part of the EDSP Tier 1 screening battery, requires the use of animals as a source of ER. Results from the BG1Luc ER TA were examined for concordance with published reports of ER binding for 34 reference substances (**Section 5.6**) There was 97% (33/34) concordance between the BG1Luc ER TA and ER binding data from the literature, and 100% sensitivity (no false negatives). In light of the excellent degree of agreement between ER binding and BG1Luc ER TA, it appears that evaluating results from BG1Luc ER TA agonist and antagonist testing may provide a viable alternative to conducting ER binding studies. This cannot currently be accomplished with the only accepted ER TA method due to the inability of the CERI STTA method to assess ER antagonist activity. ICCVAM recommends that additional validation studies should be performed to determine whether or not the BG1Luc ER TA method could replace the rat uterine cytosol ER binding assay.

Results from the BG1Luc ER TA were examined for concordance with published data from the uterotrophic bioassay (n=13 reference substances, see **Section 5.7**), which is currently listed as part of the EDSP Tier 1 screening battery. There was 92% (12/13) concordance between the BG1Luc ER TA and the uterotrophic bioassay data, and 100% specificity (no false negatives). These data indicate that the BG1Luc ER TA agonist test method has very good agreement with the *in vivo* results obtained with the uterotrophic bioassay, with no false negative results. Accordingly, ICCVAM recommends that further work be carried out to determine if the BG1Luc ER TA test method could be used in combination with other methods (to include *in vitro* metabolic activation) in a weight of evidence approach to replace the uterotrophic bioassay.

To further characterize the BG1Luc ER TA test method, ICCVAM recommends additional studies be considered:

- Additional studies/evaluations should be conducted to more completely characterize the ratio of ERα and ERβ in the BG-1 cell line and the extent to which these receptor subtypes contribute to the overall performance of the BG1Luc ER TA test method
- Additional studies/evaluations should be conducted to determine the feasibility of testing volatile substances using CO_2 permeable plastic film or other methods to seal the test plates

- Additional studies/evaluations should be conducted to determine if substances that are not soluble in DMSO could be tested in another vehicle that would more adequately solubilize the substance in culture media

- As they are identified, additional studies/evaluations should be conducted with known estrogen antagonists to expand the database of positive substances tested and thereby better characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with estrogen antagonist activity

- ICCVAM encourages users to provide all data that are generated from future studies, as they could be used to further characterize the usefulness and limitations of the BG1Luc ER TA test method as a screening test to identify substances with estrogen agonist or antagonist activity

1.4 Draft ICCVAM Recommendations: Performance Standards for the BG1Luc ER TA Test Method

ICCVAM has developed test method performance standards so that modified versions of the BG1Luc ER TA test method that are mechanistically and functionally similar can be effectively and efficiently evaluated for their validity by national and international validation organizations (e.g., the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods [ICCVAM], the European Centre for the Validation of Alternative Methods [ECVAM], and the Japanese Center for Validation of Alternative Methods [JaCVAM]) or other organizations. The ICCVAM recommended BG1Luc ER TA agonist and antagonist test method protocols are the key references used for establishing these performance standards.